CONSTRUCTION OF A NOVEL FUNGAL GUS EXPRESSION PLASMID, AND ITS EVALUATION IN *ASPERGILLUS NIDULANS*.

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ABSTRACT

A GUS expression plasmid, pFunGus, was constructed containing a multi-cloning site for the insertion of gene regulatory elements, to be used in fungal reporter gene studies. A derivative of pFunGus (pFG-gpd) was constructed by the insertion of the *gpdA* promoter (glyceradehyde-3-phosphate dehydrogenase) into the multi-cloning site of pFunGus for the assessment of the plasmid's transformation and expression properties in *Aspergillus niduans*. The correct construction of pFunGus and pFG-gpd was verified by analytical restriction digests and by its property of GUS expression in *A. nidulans*.

The plasmid was integrated into the *A. nidulans* genome via cotransformation with the phleomycin resistance plasmid, pAN8-1. Transformation frequencies of between 3 and 250 transformants per μg of pAN8-1 DNA were obtained. Initial screening for cotransformation yielded no pFG-gpd transformants. Attempts to improve cotransformation frequencies by optimisation of cotransformation conditions were unsuccessful. However, large scale screenings of transformants lead to cotransformants being isolated at a very low cotransformation frequency. Approximately 0.45% of pAN8-1 transformants possessed the GUS phenotype.

The eight pFG-gpd transformants obtained were analysed by Southern hybridisation. Six out of the eight transformants had a single copy integration. Of the remaining two transformants, one had three copies integrated at separate locations, one of which was disrupted, and the other had four copies integrated as tandem repeats, one of which was disrupted. All the transforming DNA appeared to be integrated ectopically.

The physiology of the transformants was assessed by dry weight increase, colony extension and total protein content. These showed that the transformants biology was not significantly compromised by the transforming DNA.

Finally, high levels of GUS expression were observed in all pFG-gpd transformants and the GUS expression per copy of the GUS expression cassette integrated into the genome was constant. These results showed that the transformed gene copy number determined the levels of gene activity rather than the position of integration in the genome.

Overall these results demonstrate the potential application of the versatile GUS expression plasmid, pFunGus for reporter gene studies in filamentous fungi.

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TABLE OF CONTENTS.

ABSTRACT II

ACKNOWLEDGMENTS iv

TABLE OF CONTENTS. v

LIST OF FIGURES. x

LIST OF TABLES xi

1. INTRODUCTION. 1

1.1 THE BIOLOGICAL IMPORTANCE OF FUNGI. 1

1.2 THE BIOLOGY AND GENETICS OF *ASPERGILLUS NIDULANS.* 1

1.3 TRANSFORMATION IN FILAMENTOUS FUNGI. 5

1.3.1 Overview. 5

1.3.2 Components of Fungal Expression Vectors used in Transformations. 5

1.3.3 DNA Uptake 9

1.3.4 Fate of Transforming DNA: Integration Events 10

1.3.5 Fate of Transforming DNA: Transient Transformants. 13

1.3.6 Position Effects and Gene Copy Number in Determining Levels of Transformed Gene Expression. 14

1.3.7 Cotransformation. 15

1.4 THE USE OF REPORTER GENES IN THE STUDY OF FUNGAL PROCESSES. 16

1.4.1 Overview of Methods to Study Fungal Cell Processes. 16

1.4.2 The β-glucuronidase (*gusA*) Reporter Gene System. 18

1.4.3 GUS Expression Plasmids. 19

1.5 AIMS AND OBJECTIVES OF THIS STUDY. 20

2. MATERIALS AND METHODS. 21

2.1 FUNGAL STRAINS, BACTERIAL STRAINS AND PLASMIDS 21

2.2 GROWTH MEDIA. 21

2.2.1 Liquid Media. 21

2.2.2 Solid Media. 23
2.2.3 Media Antibiotics and Supplements. 23
2.3 MAINTENANCE OF CULTURES. 24
2.4 COMMON BUFFERS AND SOLUTIONS. 24
2.4.1 TE buffer. 24
2.4.2 1 × TBE Buffer. 24
2.4.3 1 × TAE Buffer. 24
2.4.4 Tris-Equilibrated Phenol. 25
2.4.5 DNase free RNaseA. 25
2.4.6 Lysis Buffer. 25
2.4.7 Gel Loading Buffer (10 ×) 25
2.4.8 OM Buffer. 25
2.4.9 STC Buffer. 25
2.4.10 40% PEG Solution. 25
2.4.11 GUS Extraction Buffer. 26
2.4.12 10 × SSPE. 26
2.4.13 1000× Ethidium Bromide Stock (for staining of agarose gels). 26
2.5 DNA PREPARATIONS. 26
2.5.1 Phenol/Chloroform Purification of DNA. 26
2.5.2 Concentration of DNA by Ethanol Precipitation. 26
2.5.3 Boiling DNA Mini-preparation. 27
2.5.4 Large Scale Plasmid Preparation with Purification by PEG Precipitation. 27
2.5.5 Large Scale Plasmid Preparation with Purification by CsCl/EtBr Centrifugation. 28
2.5.6 Mini Plasmid Preparation for Checking Presence of Insert DNA. 29
2.5.7 Mini-preparation for Isolation of DNA from Fungal Cultures. 30
2.5.8 Extraction of DNA Fragments from Agarose Gel. 30
2.6 DETERMINATION OF DNA CONCENTRATION. 31
2.6.1 Determination of DNA concentration by spectrophotometric assay. 31
2.6.2 Determination of DNA concentration by fluorometric assay. 31
2.7 DNA MANIPULATIONS. 32
2.7.1 Restriction Enzyme Digests. 32

2.7.2 Agarose-gel Electrophoresis of DNA. 33
2.7.3 Determination of Fragment Sizes....33
2.7.4 Repairing 3′ or 5′ Overhanging DNA Ends to Generate Bunt Ends using the Klenow Fragment. 34
2.7.5 Dephosporalytion of Digested DNA 5′ Ends. 34
2.7.6 Ligation Reactions. 34
2.8 TRANSFORMATION OF *E. COLI.* 35
2.8.1 Transformation using Calcium Chloride....35
2.8.2 Transformation using Electroporation. 36
2.9 COTRANSFORMATION OF *A. NIDULANS.* 37
2.9.1 Preparation of Spore Suspensions from *A. nidulans.* 37
2.9.2 Preparation of Fungal Protoplasts. 38
2.9.3 Cotransformation of *A. nidulans* (Method 1). 39
2.9.4 Cotransformation of *A. nidulans* (Method 2). 39
2.9.5 Selection of Cotransformants. 40
2.9.6 Single Spore Purification of Transformants. 40
2.10 MEASUREMENT OF TRANSIENT EXPRESSION OF TRANSFORMED DNA. 40
2.11 DNA HYBRIDISATIONS. 41
2.11.1 Southern Blotting. 41
2.11.2 Radioactive (^{32}P) Labelling of DNA Probe. 42
2.11.3 Southern Hybridisation. 43
2.11.4 Autoradiography of Southern Blots. 44
2.12 GROWTH RATE DETERMINATION OF FUNGAL TRANSFORMANTS. ..44
2.12.1 Colony Growth. 44
2.12.2 Dry Weight Determination. 44
2.13 MEASUREMENT OF GUS EXPRESSION. 45
2.13.1 Protein Quantification. 45
2.13.2 Quantification of GUS Expression. 46
2.14 STATISTICAL ANALYSIS. 47
2.14.1 Linear Regression, Correlation Coefficient, and Exponential Regression. 47
2.14.2 Z-test. 48

3. RESULTS. 49

3.1 CONSTRUCTION OF A GUS EXPRESSION PLASMID, pFUNGUS. 49

3.1.1 Preparation of DNA Fragments Required for pFunGus Construction. 49

3.1.2 Ligation of DNA Fragments for pFunGus Construction.....53

3.1.3 *E. coli* transformation of Ligated Fragments and Preliminary Analyses of Transformants. 53

3.1.4 Identification and Confirmation of Potential pFunGus Containing *E. coli* Transformants. 54

3.2 CONSTRUCTION OF PFG-GPD, A PROMOTER CONTAINING DERIVATIVE OF PFUNGUS. 58

3.2.1 Preparation of DNA Fragments Required for pFG-gpd Construction. 58

3.2.2 Ligation of DNA Fragments for pFG-gpd Construction. 60

3.2.3 *E. coli* Transformation of Ligated Plasmid and Preliminary Analysis of Transformants. 61

3.2.4 Identification and Confirmation of Potential pFG-gpd Containing *E. coli* Transformants. 61

3.3 TRANSIENT EXPRESSION OF THE PFG-GPD PLASMID. 67

3.3.1 Transient GUS Expression Levels in *A. nidulans* Transformants.....67

3.4 COTRANSFORMATIONS OF *A. NIDULANS*. 70

3.4.1 Preparation of Transforming DNA for all Cotransformations. 70

3.4.2 Initial Cotransformations of *A. nidulans* R21. 71

3.4.3 Attempt at Optimisation of Cotransformation Procedure using pNOM102. ...75

3.4.4 Large Scale Cotransformations of *A. nidulans* Yielding Cotransformants with pFG-gpd + pAN8-1.....77

3.4.5 Selection and Purification of Cotransformants. 79

3.5 ANALYSIS OF PFG-GPD *A. NIDULANS* TRANSFORMANTS.....80

3.5.1 Analysis of Integration of pFG-gpd into the *A. nidulans* Genome.....80

3.5.2 Physiological Characteristics of *A. nidulans* Transformants. 89

3.5.3 Analysis of Total Protein Levels in Transformants at Mid Log Phase. 92

3.5.4 Analysis of GUS Expression in pFG-gpd Transformants. 96

4. DISCUSSION. 99

4.1 OVERVIEW. 99

4.2 EVALUATION OF COTRANSFORMATION. 99
4.2.1 Possible Reasons for Low Levels of Cotransformation Frequencies of pFG-gpd. 99
4.2.2 Possible Explanations for Higher Cotransformation Frequencies Observed with pNOM102 than with pFG-gpd. 103
4.3 EVALUATION OF INTEGRATION EVENTS IN TRANSFORMANTS. 104
4.3.1 A Predominance of Single Copy Integrations with Intact GUS Expression Cassettes Observed in pFG-gpd Transformants. 104
4.3.2 Analysis of Ratios Between Type I / II / III Integration Events Show a Predominance of Type II (Ectopic) Integration Events. 105
4.3.3 Physiological Response of *A. nidulans* to the Presence of pFunGus Derivatives. 106
4.3.4 Correlation Between GUS Expression Levels and Transformed Gene Copy Number. 106
4.4 SUMMARY AND FUTURE PROSPECTS. 108

LIST OF FIGURES.

Figure 1 Life cycle of *Aspergillus nidulans,* (reproduced from Pontecorvo, 1953).....3

Figure 2 Schematic representation of types of expression cassettes.....8

Figure 3 Diagram of the intergration events observed in fungal transformation.....11

Figure 4 Summary of pFunGus Construction.....50

Figure 5. Agarose gel showing the profile of bands produced for analysis of the potential pFunGus clone.....54

Figure 6 Restriction map of pFunGus.....59

Figure 7 Agarose gel showing the profile of bands produced for analysis of the potential pFG-gpd clones.....62

Figure 8 Restriction map of pFG-gpd.....66

Figure 9 Agarose gels showing the profile of bands produced by digesting *A. nidulans* DNA to demonstrate consistency in the DNA concentration and degree of digestion between lanes.....82

Figure 10 Profiles of the integration of the GUS cassette in pFG-gpd transformants.....84

Figure 11 Diagrammatic representation of the types of integrations observed. 87

Figure 12 Graphs of colony extension versus time.....90

Figure 13 Growth of untransformed and transformed *A. nidulans* in liquid media defined as dry weight.....93

LIST OF TABLES

Table 1 Plasmids and Strains.....22

Table 2. Summary of band sizes produced from digests for the analysis of the potential pFunGus clone.....57

Table 3 Summary of band sizes produced from digests for the analysis of the potential pFG-gpd clones.....64

Table 4 GUS enzyme specific activity in transformed protoplasts[a]. 69

Table 5 Results of initial cotransformation experiments.....73

Table 6 Summary of cotransformations with pNOM102 and pAN8-1. 76

Table 7 Summary of results for the final cotransformation experiments. 78

Table 8 Summary of the types of integrations. 86

Table 9 Growth rates of transformants determined by dry weight versus time. 94

Table 10 Protein concentrations in GUS extracts of transformants in mid-log phase of growth.....95

Table 11 Specific activities of GUS Enzyme in pFG-gpd transformants showing the correlation with copy number.....98

1. INTRODUCTION.

1.1 THE BIOLOGICAL IMPORTANCE OF FUNGI.

Fungi are eukaryotic organisms that have provided model systems for studying cell processes, as stated by Watson *et al.*, 1987 in a text on molecular biology: "If we can solve the same problem equally well with yeast or with human cells, common sense tells us to stick with the simpler less expensive system". This thinking led to a wealth of research using fungi, and headed an understanding of basic processes in eukaryotic cells, in particular of meiosis and mitosis. New research developments have enabled studies in animal and plant systems to become increasingly viable, therefore research of fungi has turned to studying them in there own right as unique life forms, for their contribution to the ecosystem, and importance medically and economically (Wessels, 1991).

Fungi obtain organic nutrients by either infecting other living organisms as parasites, or by breaking down dead organic matter as saprophytes (Alexopoulos and Mims, 1979). They are responsible for the majority of known plant diseases as well as many diseases of human and animals (Wessels, 1991). They also provide the basis for many industrial processes, including commercial fermentations for organic acid production (e.g. citric acid) and in the food industry for the preparation of cheeses. The health industry depends on fungi for the production of drugs, for example corgmentrine and cortisone, vitamin preparations, and finally the manufacture of a number of antibiotics, e.g. penicillin and cephalosporin (reviewed by Davies, 1991; Martin, 1991).

1.2 THE BIOLOGY AND GENETICS OF *ASPERGILLUS NIDULANS.*

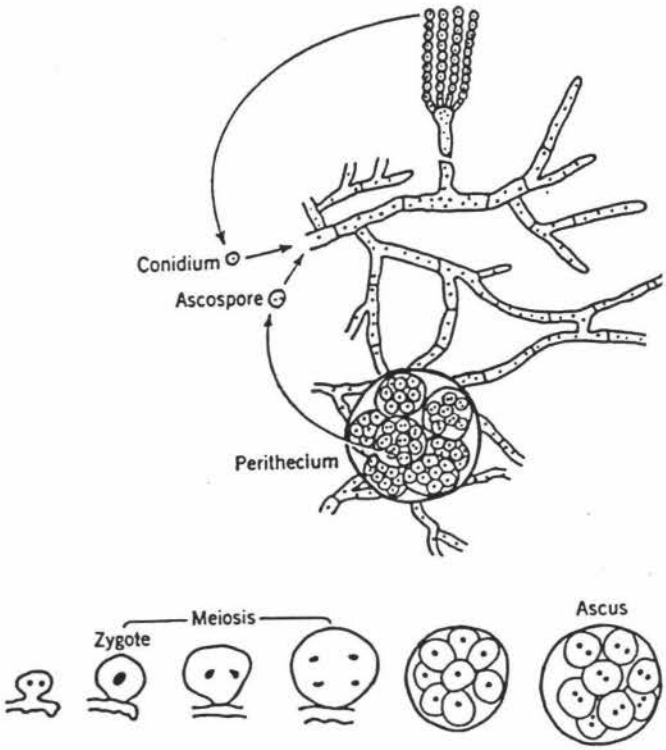
Aspergillus is a widely distributed genus that plays a major role in the natural world as well as in human processes, positively in industry for the production of secondary metabolites and negatively in causing disease in plants, animals and spoilage of foods (Martinelli, 1994). The species *A. nidulans* has been utilised as a model system for the

study of biological and genetic processes in filamentous fungi. Several features of *A. nidulans* make it suitable for study as a model organism (Pontecovo, 1953), and the following discussion will describe *A. nidulans* from this perspective.

Aspergillus nidulans is a filamentous fungi which develops as hyphae which are collectively known as mycelia. Hyphae have cell walls enclosing a cytoplasmic mass which moves inside the tubes. As the cell wall is built at the tip the cytoplasmic mass moves forwards and ramifies into branches (Wessels, 1991). In *A. nidulans* hyphae are septate with multi-nucleate compartments. Undifferentiated hyphae may form structures that produce asexual spores that can then germinate and produce more hyphae. The asexual stage is prevalent in *A. nidulans* and produces spores, (conidia) that contain a single haploid nucleus. The sexual stage can occur by the fusing of two hyphae, either from different or the same mycelia making *A. nidulans* is a homothallic species. Sexual recombination results in meiosis and the production of a multi-cellular structure, an ascus, containing four diploid ascospores. This defines *A. nidulans* as a member of the fungal class, Ascomycetes (Martinelli, 1994). This life cycle is shown in Figure 1.

Aspergillus nidulans has minimal nutritional requirements, and prefers to grow at temperatures around 37-40°C. At 37°C it grows relatively rapidly with a rate of hyphal extension of approximately 4μm per minute on complete or minimal media (Pontecorvo, 1953). Conidia lend themselves to plating techniques used for yeast and bacteria. Rapid growth generally means conidia are available for harvest after 48 hours, and colonies are classifiable by morphology, colour of conidia, etc., thus allowing quick analysis of many generations. Tetrad analysis can be applied to the ascospores produced by the sexual stage and these ascospore have been utilised for the long term storage of strains (Pontecorvo, 1953).

Figure 1 Life cycle of *Aspergillus nidulans,* (reproduced from Pontecorvo, 1953).



The genetics of filamentous fungi has been well studied, especially in *A. nidulans*. This species, relative to other fungi, has a small genome of approximately 20,000kb, about 7 times the size of *E. coli* and 1% the size of a human haploid genome. The genome is organised on 8 chromosomes per haploid genome (reviewed by Turner, 1993). Unique sequences make-up 97-98% of the total genome, with the remaining 2-3% repeated sequences being assigned to the ribosomal RNA genes. The 5′ region of a typical gene carries sequences responsible for the binding of regulatory proteins and general transcription factors. Many genes are strongly regulated by activation and/or repression at the level of transcription (reviewed by Caddick and Turner, 1993). A feature of fungal genome organisation seen in *A. nidulans* is functionally related genes occurring in clusters. These contain two or more loci that code for related functions, examples of these are the loci involved in; alcohol utilisation (Lockington *et al.*, 1985), penicillin biosynthesis, (McCabe *et al.*, 1990), and nitrate utilisation (Johnston *et al.*, 1990). Of the 82+ genes isolated by cloning, many have been sequenced permitting studies of their structure and function (reviewed by Matinelli *et al.*, 1994). For these reasons *A. nidulans* provides a parallel system for the study of processes in more complex and less well defined fungi.

Methods for transforming sequences into *A. nidulans* are well established (Tilburn *et al.*, 1983; Ballance *et al.*, 1983; Yelton *et al.*, 1984). The DNA is integrated into the genome and produces mitotically stable transformants. The multi-nucleate nature of hyphae means balanced heterkaroytes can be created (Pontecorvo, 1953). These contain a mixture of two parental nuclei that differ at one or more genetic loci, and provide the opportunity create and investigate lethal mutations. Transformation has provided an opportunity to study gene expression and regulation at the molecular level. This is discussed in more detail in Section 1.3.

In summary *A. nidulans* provides a good model system for the study of biological and genetic phenomena in filamentous fungi. It is (i) rapidly growing, (ii) its genome organisation is relatively simple with little repeating DNA and (iii) regulation of gene expression predominantly occurring a the level of transcription, (iv) many control and structural genes have been cloned and sequenced, and (v) transformation systems have been established.

1.3 TRANSFORMATION IN FILAMENTOUS FUNGI.

1.3.1 Overview.

Transformation is an experimental method for introducing DNA sequences into genomes. It was first reported in filamentous fungi (*Neurospora crassa*) in 1973 (Mishra and Tatum, 1973), and now is widely established in many types of fungi. Development of transformation systems has not only allowed the introduction of endogenous sequences into fungi, but the transfer of genes between fungal species and from prokaryotes, animal, and plants into fungi. This is termed 'heterologous expression', and predominantly these genes are transcribed, translated, and give functional products. Many gene products of bacteria, plant and animal origin have been produced under the control of fungal regulatory systems, and provide many applications (reviewed by Saunders *et al.*, 1989; Davies, 1994). Also, regulatory systems are transferable between Ascomycetes. This shows conservation of regulatory mechanisms within this class of filamentous fungi (Austin *et al.*, 1990; Couteaudier *et al*, 1993).

There is extreme flexibility in the genes that can be expressed in *A. nidulans* (reviewed by Davies, 1994; Goosen *et al.*, 1992). These include; vertebrate proteins for pharmaceuticals, other fungal proteins for industrial and research purposes, bacterial proteins for selection systems (Section 1.3.2.1) and insect proteins, as reporter genes for the study of gene expression (reporter genes are discussed in Section 1.3.7).

1.3.2 Components of Fungal Expression Vectors used in Transformations.

Vectors used for transformation in filamentous fungi contain, (i) the expression cassette containing the DNA sequence to be expressed attached to fungal regulatory elements, (ii) a bacterial origin of replication, (iii) bacterial selectable marker for cloning purposes e.g. ampicillin resistance, and may also contain (iv) a fungal selectable marker. (The latter can also be supplied on a separate vector though the process of cotransformation; Section 1.3.7) In the following section some of the vector components will be described (reviewed by Turner, 1994).

1.3.2.1 Selectable Markers in A. nidulans.

Several types of selectable markers are available for fungi. These are either auxotrophic selectable markers, or dominant selectable markers (reviewed by Goosen *et al.*, 1992; Turner, 1994). Auxotrophic markers require the complementation of a phenotype in a mutant strain. For example, an *A. nidulans* strain with a mutation in the orotidine-5'-phosphate decarboxylase (*pyrG*) gene will only grow when media is supplemented with uracil or uridine. When transformed with the *pyrG* gene, transformants can be selected on media lacking uracil (Oakley *et al.*, 1987). The drawbacks in using auxotropic markers are the requirement for the appropriate mutation in the recipient strain, and that the mutant strains are often compromised even when supplied with the required substrate (Turner, 1994). Therefore these transformants do not truly represent the wild type genetic background and it is often more desirable to select on the basis of a dominant selectable marker.

Fungal antibiotic resistance markers have been difficult to obtain due to the variable sensitivity of filamentous fungi to antibiotics commonly used in yeast and higher eukaryotes (Goosen *et al.*, 1992). Therefore dominant selectable markers have been created by fusing bacterial antibiotic genes to fungal expression signals. These hybrid-dominant selectable markers have been constructed to confer resistance to hygromycin B (Punt *et al.*,1987), and phleomycin (Mattern *et al*, 1988) for example. Phleomycin resistance is becoming the dominant selectable marker of choice due to *A. nidulans'* sensitivity to it. Little or no growth is seen in the wild type strain at antibiotic concentrations of greater than 10μg per ml of growth medium (Punt *et al.*, 1992). Phleomycin is a metalloglucopeptide antibiotic causing DNA strand scission, and the resistance gene from *Streptomyces hygroscopicus* produces a product that binds and inactivates the antibiotic (Malpartida *et al.*, 1983).

1.3.2.2 The Expression Cassette.

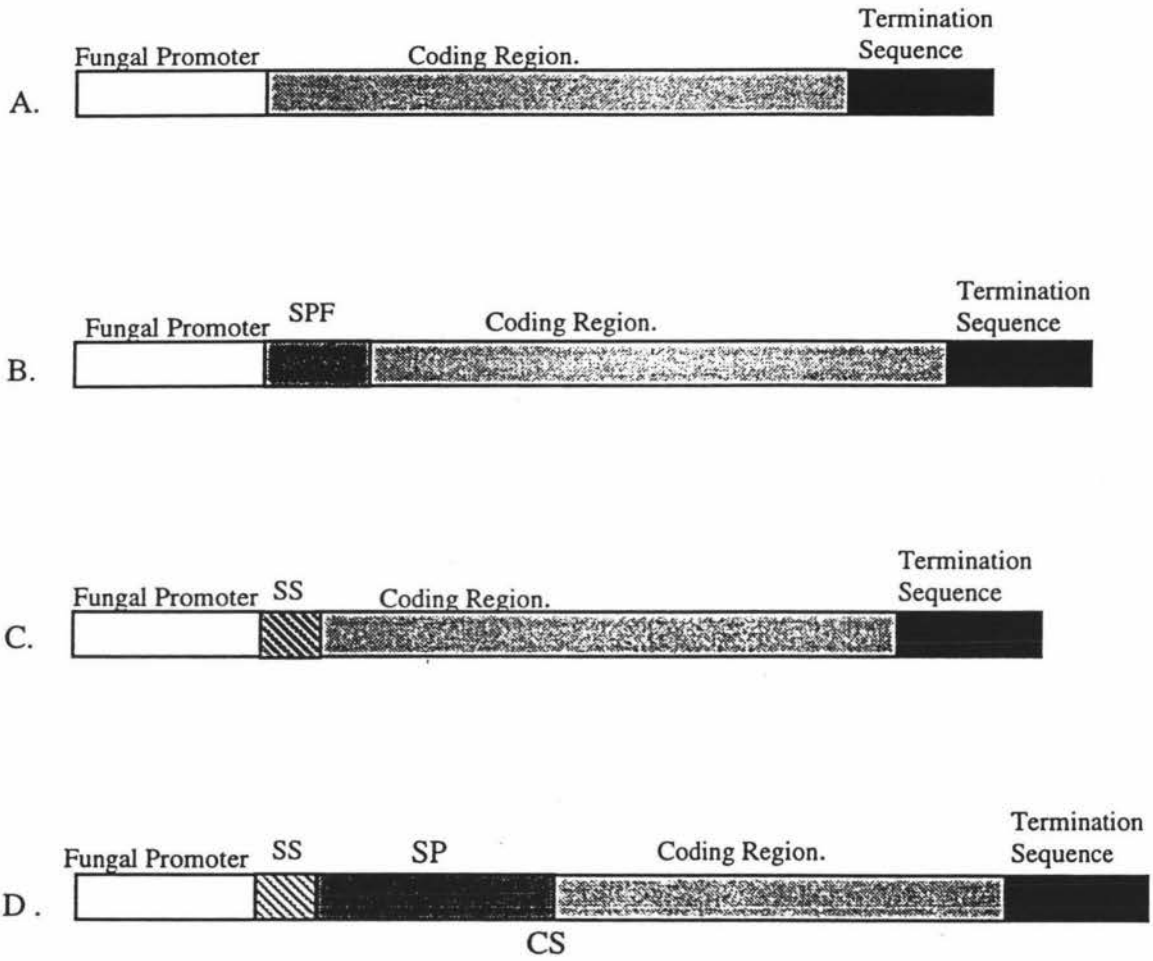
A simple expression cassette contains; a fungal promoter (or regulatory element), the coding region, and the fungal transcription stop sequence. For the expression of heterologous genes the expression cassette may also contain a sequence for a stabilising

protein, a signal sequence e.g. for secretion, or other processing sequences such as a cleavage sequence (Davies, 1994). The types of expression cassette constructs are shown in Figure 2.

In *A. nidulans* several promoters have been used in expression cassettes. The choice is generally based on whether a constitutive or inducible promoter is required. Most promoters used originate from genes involved in carbon-source utilisation pathways, as their expression tends to be high (reviewed by Davies, 1994). Constitutive promoters are generally more convenient because inducible conditions are not required and the product is obtained faster. Examples of constitutive promoters are glyceraldehyde-3-phosphate dehydrogenase (*gpdA*; Punt, 1990), and triose phosphate isomerase (*tpi*; McKnight *et al.*, 1986). However, if the protein to be produced is not stable, is toxic to the cell, or the product needs to be produced at a certain developmental stage, then inducible promoters can be used. Under the control of these promoters gene expression is turned on by a chemical or environmental trigger. Commonly used examples of these are the promoters of acetamidase (*amdS;* Turnbull *et al.*, 1989), and alcohol dehydrogenase I (*alcA;*Gwynne *et al.*, 1987). Finally, parts of promoters and regulatory elements have been used for the assessment of these elements in driving gene expression. For example, the promoter of the *A. nidulans* isopenicillin N synthase gene has been dissected by this method (Pérez-Esteban *et al.*,1993) .

All eukaroytic genes contain a conserved sequence in the region immediately surrounding the translational start codon, called the Kozak sequence (Kozak, 1987). This is involved in the recognition and binding of the gene expression machinery. If heterologous genes being expressed are not of eukaroytic origin then they are often modified to contain this sequence (Ballance, 1991).

Figure 2 Schematic representation of types of expression cassettes.



A and B are cassettes designed for the expression of intracellular gene products, while C and D are cassettes designed for secreted gene products (Section 1.3.2.2). SPF, stabilising protein fusion; SS, signal sequence for secretion; SP, secreted protein coding sequence; CS, endogenous site for enzymatic cleavage of unrequired sequences. This diagram presents constructs described by Davies (1994).

Finally, transcriptional terminators are required for the correct termination and processing of the mRNA product (Devchand and Gwynne, 1991).

1.3.3 DNA Uptake.

Transformation procedures are well established in *A. nidulans* (Tilburn *et al.*, 1983; Ballance *et al.*, 1983; Yelton *et al.*, 1984). The most commonly used and most successful transformation techniques involve preparation of osmotically sensitive cells by the removal of the fungal wall by cell wall-digesting enzymes. These protoplasts are subsequently treated with $CaCl_2$, polyethylene glycol (PEG), and exogenous DNA. After an incubation period the mixture is diluted and aliquots spread on stabilised selective media, either directly or in a soft agar overlay (methods reviewed by Goosen *et al.*,1992; Fincham, 1989). Filamentous fungi do not have non-chromosomal replicons with high copy numbers, such as those used in yeast and bacteria, therefore transformation relies on integration of the DNA into the genome (Davies, 1991). Three types of integration events are observed (Hinnen *et al.*, 1978), and these are discussed in detail in Section 1.3.4.

A model of DNA uptake during transformation is the 'competent cells' model that predicts that not all cells are capable of DNA uptake, but that those that are will take up multiple DNA molecules (Grotelueschen and Metzenberg, 1995). Evidence from cotransformation experiments (Section 1.3.4) heavily favours the 'competent cells' model as cotransformation with two separate vectors occurs at a high frequency (Austin and Tyler, 1990; Wernars *et al.*, 1987).

If only a certain number of cells are competent, what keeps the rest incompetent? Grotelueschen and Metzenberg (1995) hypothesised that this could be a feature of either permeability of the cell membrane or the characteristics of the nuclei. They showed that the requirement for competence is at the level of the nucleus. They then hypothesised that the limiting event in transformation is the availability of suitable sites for integration. The nuclei in a protoplast population divide asynchronously, and therefore there may be only a narrow window in the nuclear cycle in which the genome is available to be transformed. It was shown by Contour-Clamped Homogeneous Electric

Field (CHEF) gel electrophoresis and Southern hybridisation analysis that there are competent chromosome sites for ectopic integration (Grotelueschen and Metzenberg, 1995). This followed hypothetical models proposed in mammalian and yeast cells that ectopic integration may occur at pre-existing breaks in the chromosome (Roth and Wilson, 1988; Schiestl *et al.*, 1993).

The hypothesis that the limiting event in transformation is DNA integration is corroborated by transient expression studies (Mönke and Schäfer, 1993). Research showed that the frequency of transformation resulting in transient expression levels in protoplasts far exceeded the frequency of stable transformation. Also that at least 99% of transformants lose the transforming DNA. This implies that the limiting factor in transformation is DNA integration rather than DNA uptake (Mönke and Schäfer, 1993).

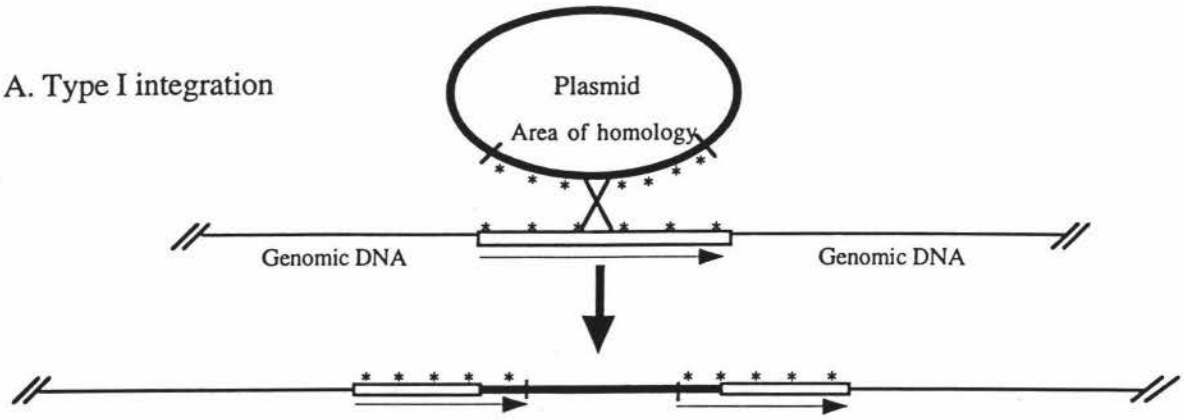
1.3.4 Fate of Transforming DNA: Integration Events.

Stable transformation of *A. nidulans* requires the integration of the exogenous DNA into the genome. Three types of integration were first defined in yeast by Hinnen *et al.* (1978), and those observed in filamentous fungi are very similar (reviewed by Goosen *et al.*, 1992; summarised in Figure 3). Type I integration occurs at a homologous site resulting from a single crossover event between homologous sequences present in the plasmid and the genome. This results in a linked duplication of the sequences separated by the plasmid DNA. Type II integration occurs at a heterologous sites in the genome. It is called 'ectopic integration' and results from a single cross-over event between two non-homologous sequences. With both these types of integration the transformation can contain one or more copies arranged in a tandem array. Finally, type III integration occurs a homologous site resulting in a double cross-over event between the homologous sequences. This is termed gene conversion or replacement and results in the plasmid sequence replacing the sequence in the genome. Methods have been developed using type III integration for the deletion, mutation, and cloning of genes (e.g. Miller *et al*, 1985; Wernars *et al*, 1987). All these integration types have been shown to occur simultaneously in a single fungal transformant (Yelton *et al.*, 1984).

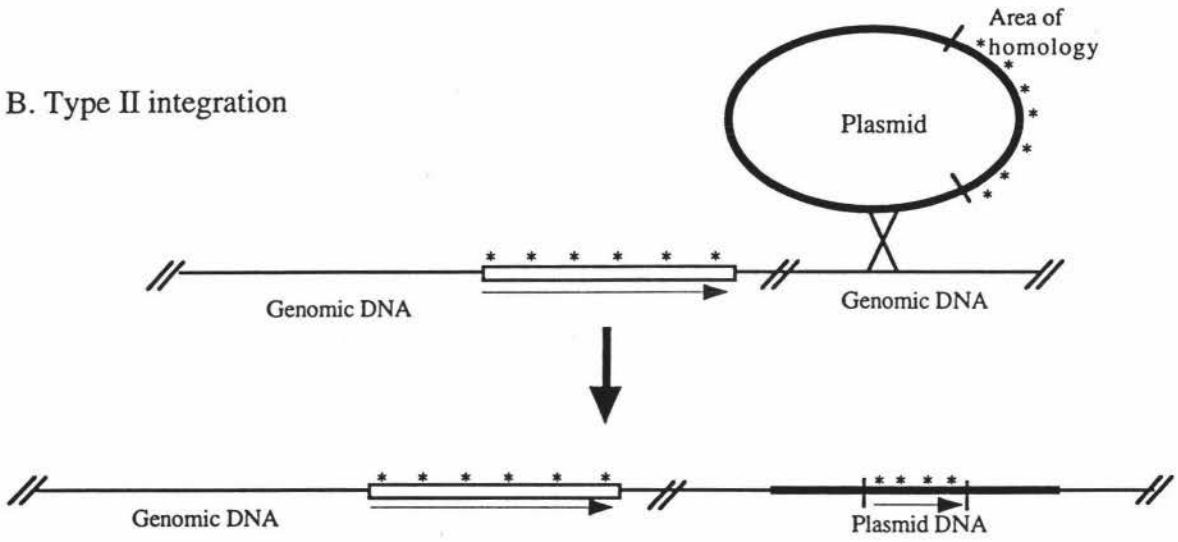
Figure 3 Diagram of the intergration events observed in fungal transformation.

Type I integration results in integration at a homologous site, type II results in ectopic integration and type III results in sequence replacment (Section 1.3.4). The diagram represents the types of integration events described by Hinnen et al, (1978).

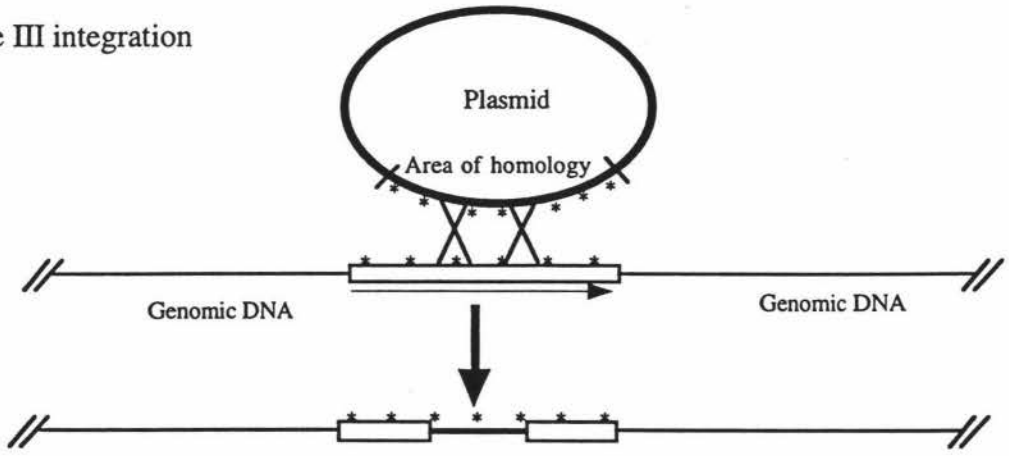
A. Type I integration



B. Type II integration



C. Type III integration



The frequencies with which the different types of integration will occur in a given transformant are difficult to predict as they vary depending on the composition of the vector, the nature of the selective marker, the form in which the vector is used (circular or linear), and the genetic background of the recipient strain. However one general rule is that the number of multi-copy transformants can be increased by increasing the total amount of DNA (reviewed by Goosen *et al.*, 1992).

1.3.5 Fate of Transforming DNA: Transient Transformants.

In transformation experiments two types of transformants are commonly observed. The first type grow strongly and spore well on selective media, the second type present weaker growth and fail to grow when transferred to fresh selective media. The latter type has been classified as abortive transformants (Tilburn *et al.*, 1983). It is hypothesised that the molecular basis of this is transient expression of the transformed vector. The vector is not integrated into the genome and is lost during cell division. The product of the transient expression may confer the transformed phenotype until it is titrated out by mitosis. Transient expression can be used to investigate gene expression without the need for stable integration. This method saves time, circumvents the problems of multi-copy integration and position effects (Section 1.3.6), and is useful for establishing transformation techniques and for rapid evaluation of expression signals (Mönke and Schäfer, 1993; Judelson *et al.*, 1993).

Stable expression requires integration into the genome and once this has occurred stability remains through many rounds of sub-culturing on non-selective media (Yelton *et al*, 1984). However meiotic instability has been observed in some filamentous fungi, and has been extensively studied in *N. crassa*. It has been shown to be due to gene inactivation by methylation, and is termed RIP - Repeat Induced Point Mutation (Selker, 1990). RIP seems limited to a subset of filamentous Ascomycetes, and has not been reported in *A. nidulans*, although other forms meiotic instability, for example unequal recombination, may still occur (Goosen *et al*, 1992).

1.3.6 Position Effects and Gene Copy Number in Determining Levels of Transformed Gene Expression.

Once an expression cassette has integrated into the genome levels of transformed gene expression may still vary depending on the number of copies of the cassette introduced and the area of the genome that the cassette is introduced into (position effects; Turner, 1994). There are two sources of position effects. Firstly, the transformed gene may integrate downstream of promoter elements. These elements may be positively or negatively regulated, and therefore will have an additive effect with the transformed regulatory elements to alter the expression of the transformed gene . Secondly, the region of the genome that the DNA integrates into may be transcriptionally active or inactive, and may vary in activity depending on the developmental stage or on external stimuli (Couteaudier *et al.*, 1993). It has been clearly shown that position effects play a large role in the expression of transformed genes in animals and plants, while they play a very small role in bacteria (Martinelli, 1994). The significance of position effects seems to climb as the complexity of the genome increases and the genome contains larger amounts of repeated DNA sequences.

In keeping with this is the observation that in fungi the strength of position effects is connected with the genome complexity. For example in the position effects are significant in the fungal class, Oomycetes, although to a lesser extent than plants and animals (Judelson *et al.*, 1993). The genome of Oomycetes' is more complex than the genome of the Ascomycetes', also, *A. nidulans* has a relativity simple genome with only 2-3% consisting of repeated sequences (Mao and Tyler, 1991; Section 1.2). Therefore compared to Oomycetes, animals and plants, position effects may be expected to play a less significant role in determining the levels of expression of a transformed gene. This has been demonstrated by a correlation between an increase in copy number and an increase in hygromycin resistance (Punt *et al.*, 1987) in *Aspergillus* species. In the production of heterogeneous gene products, e.g. human interferon, high copy number also gave increased yields of the product (Gwynne *et al.*, 1987). A comprehensive study of the activity of interferon α-2 under the control of *alcA* (alcohol dehydrogenase) expression signals was carried out for transformants containing different copy numbers

of the expression cassette. The importance of the copy number of the *alcR* (alcohol dehydrogenase regulatory gene) was also analysed. It was concluded that expression increased in a roughly linear fashion with the number of copies present up to a number of 7-10 copies. It was hypothesised that when the *alcA* copy number reached 10 copies the *alcR* product was titrated out. This was corroborated by experimental evidence showing that the multi-copy *alcR* transformants continued the linear relationship up to 20 copies. Therefore, in this case copy number showed a linear relationship with yield (Davies, 1991). However, in another Ascomycetes, *Fusarium oxysporum*, it was concluded that position effects were more important than gene copy number. This was based on the comparison of two single copy transformants, one single copy transformant showed enzyme activity 80-fold higher than another single copy transformant. Plasmid rescue and sequence analysis indicated that the highly expressing single copy transformant had integrated downstream of a recognisable enhancer element (Couteaudier *et al.*, 1993). Studies on the relative effects of copy number and position are complicated due to the difficulty in determining the number of active copies of the cassette accurately.

1.3.7 Cotransformation.

When a sequence is introduced into an organism, it may be a disadvantage to need to physically link the sequence to a selectable marker. However, introduction of a vector containing a non-selectable sequence can be achieved by dual transformation with a vector containing a selectable gene, without the need for prior covalent linkage of the two vectors. This process is called cotransformation, and it has been established in *A. nidulans* (Wernars *et al.*, 1987). Cotransformation occurs since not all protoplasts in a preparation are available to be transformed, however a single protoplast that is competent can transform more than one plasmid simultaneously (Grotelueschen and Metzenberg, 1995; discussed in Section 1.3.2).

This principle was first demonstrated by Wernars *et al.* (1987), *A. nidulans* was cotransformed with vectors containing the *amdS* gene and *trpC* gene. Each vector was initially selected for, and transformants screened for the presence of the second vector. Results showed that the cotransformation frequencies varied depending on which vector

was initially selected for; 95% of the *amdS*$^+$ transformants were also *trpC*$^+$, while only 62% of the *trpC*$^+$ transformants were *amdS*$^+$. This was hypothesised to reflect a relatively large number of *amdS* genes becoming inactivated during the act of integration, since similar results were obtained with a plasmid containing both the genes together. This shows that the frequency of cotransformation is dependent on the nature of the selection system. It was also observed that cotransformation frequencies increased with increases in the total amount of cotransforming vectors, and increases in the ratio of cotransforming vector to transforming vector (containing the selectable marker).

Since these initial experiments cotransformation has been extensively used to introduce a variety of genes for the production of heterologous gene products in industry, e.g. *E. coli* enterotoxin subunit B (Turnbull *et al.*, 1989) and human interferon α-2 (Gwynne *et al*,1987) and reviewed by Saunders *et al.*, 1989. Also for the study of gene expression by the introduction of the reporter genes e.g. β-galactosidase (*lacZ*; Van Gorcom *et al.*, 1986) and β-glucuronidase (*gusA*; Roberts, *et al.*, 1989) (discussed in Section 1.3.7). Most papers report cotransformation as a percentage of the transformants, of above 60%. In a study by Roberts *et al.*, (1989) it was reported that when cotransforming with vector containing the *gusA* gene and the selectable marker *argB*, they reported cotransformation frequencies "up to 50%".

1.4 THE USE OF REPORTER GENES IN THE STUDY OF FUNGAL PROCESSES.

1.4.1 Overview of Methods to Study Fungal Cell Processes.

Through microscopy, with or without chemical staining procedures, it is possible to see cells and the organelles within them, but this gives only limited information about the metabolic processes that are occurring. A classical path for the elucidation of fungal cell metabolic processes has used a combination of classical genetic techniques for the creation of mutants, and the characterisation of these mutants by biochemical analysis. In *A. nidulans*, examples of metabolic processes that have been studied this way are; carbon

metabolism, alcohol metabolism, inorganic nitrogen assimilation, sulphur metabolism etc (reviewed in Martinelli and Kinghorn, 1994). A question not answered by this type of biochemical analysis is how these processes are spatially organised in the fungal cells. Recent staining techniques utilise fluorochromes, dyes that are absorbed by cell organelles or bind to specific residues on or inside the cells (reviewed by Butt *et al.*, 1989). Upon binding to their target these produce a fluorescence that is detectable through a microscope. In mycology, this has revealed much information about cell processes, and the differentiation and growth of the hyphae. However, these dyes have disadvantages. Their cytological use is rather limited, they are only available for certain cell types under certain conditions, and it is not known what damage they or the cell fixing process required inflicts on the cell (Yuan and Heath, 1990). Recent molecular genetic techniques have been developed that allow the study of specific cell processes by both visual observation and biochemical assay. This involves the linking the promoter of a gene involved in a process of interest to a reporter gene (Bardonnet, 1988).

A reporter gene is a gene whose product is easy to detect and assay. When fused to expression signals from a gene of interest, the reporter gene expression reflects the expression characteristics determined by those signals in the original gene (Jefferson, 1987). There are several considerations when using reporter genes in fungi. For analysis of expression signals downstream from the promoter the reporter gene may need to be fused in frame with the protein-coding region of the gene of interest, and some processing will not occur as for the endogenous protein. The reporter gene product will not be transported in the same manner as the endogenous protein, although the fusing of a signal peptide may alleviate this. Because fungal vectors must integrate into the genome, the level of reporter gene activity may not only reflect the activity conferred by the expression signals, but also will be influenced by position effects and gene copy number (Section 1.3.6). Another consideration is the stability of the reporter gene product. A stable gene product may be necessary for some applications, but for quantitative measurements, the levels of reporter gene activity may not represent the levels of promoter activity at that time but the accumulation of the reporter gene product over development. This problem can be circumvented by using inducible promoters (Section 1.3.2).

The first reporter gene used in filamentous fungi was the *E. coli* β-galactosidase (*lacZ*). This system is commonly used in bacteria and plants (reviewed by Silhavy and Beckwith, 1985), and was experimented with in *A. nidulans* (van Gorcom *et al.*, 1985; 1986). In *A. nidulans, lacZ* fusions have facilitated the molecular characterisation of promoters and their individual elements, such as the promoters of 3'-phosphoglycerate kinase (Streatfield *et al.*, 1992) and isopenicillin-N-synthetase (Pérez-Esteban *et al.*, 1993). However, the sensitivity of this system is low due to the endogenous β-galactosidase enzyme activity that is present in many fungi (Roberts *et al*, 1989).

1.4.2 The β-glucuronidase (*gusA*) Reporter Gene System.

Recently attention has focused on an alternative reporter gene, the *E.coli uidA* (or *gusA*) gene. Encoding β-glucuronidase, a hydrolase enzyme (Jefferson, 1987). It has been developed as a powerful tool for the assessment of gene activity in transgenic plants and for the development of molecular genetic analysis of plants. This system is effective in plants as there is no endogenous enzyme activity, and both histochemical and fluorometric substrates are available (Jefferson, 1987). These substrates are cleaved by the GUS enzyme to give a product that can be qualitatively or quantitatively detected. Another attractive feature of the *gusA* gene system is that its product is able to tolerate large amino-terminal additions, therefore construction of translational fusions are feasible and provide a valuable method for assaying the behaviour of transit or signal peptides (Jefferson, 1987). Also, with the appropriate signals the GUS enzyme can be translocated across chloroplast membranes with high efficiency indicating an ability to be translocated across all cell membranes (Jefferson, 1987).

The use of *gusA* as a potential reporter gene in fungi was initially studied by Roberts *et al.* (1989). In this study, *A. niger, A. nidulans*, and *Fulvia fulva* were transformed with the *gusA* gene driven by the *A. nidulans* promoter, *gpdA*. Histochemical staining of mycelia and assays of cell extracts with a fluorometric substrate showed that the *gusA* gene had been expressed. Unlike *lacZ* fusions in fungi, no significant endogenous GUS activity was observed. Since these initial studies *gusA* systems have been set up in other fungi, e.g. the plant pathogens *Pseudocercosporella herpotrichoides* (Bunkers, 1991), and *Fusarium*

oxysporum (Couteaudier *et al.*, 1993). The *gusA* reporter gene system has been used in several applications. GUS activity was demonstrated in perennial ryegrass infected with *gusA* transformed *Acremonium* endophyte. This demonstrated the use of a surrogate host for the introduction of foreign genes into perennial ryegrass (Murray *et al.*, 1992). Transient expression of transformed genes has been demonstrated in *Cochliobolus heterosrophus* using *gusA* (Mönke and Schäfer, 1993). The GUS reporter gene system has been used to demonstrate lack of conservation of gene expression machinery between the classes of fungi, Ascomycetes and Oomycetes (Judelson, 1992; Pieterse *et al.*, 1995). Finally, *gusA* fusions to the *adh1* (alcohol dehydrogenase) and *ver1* (aflatoxin biosynthesis gene) promoters of *A. flavus* have allowed the evaluation of transcriptional control and induction of aflatoxin production *in situ* (Woloshuk and Payne, 1994; Flaherty *et al.*, 1995).

1.4.3 GUS Expression Plasmids.

The components of GUS expression vectors are essentially the same as those for the expression of other heterologous genes (see Section 1.3.2). The GUS expression cassette is the most important component in the expression vector. It includes the *gusA* gene, transcriptional regulatory sequences, and sequences required for the termination and processing of the RNA transcript (Devchand and Gwynne, 1991). Because of the application GUS expression vectors to studying and comparing many promoter elements, a basic GUS vector without the expression sequences, and with a multi-cloning site for ease in cloning in these sequences can be initially constructed (this study).

A popular promoter for driving *gusA* expression when systems are being developed is the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (e.g. Roberts *et al.*, 1989; Bunkers, 1991). This is a gene from the glycolytic pathway, therefore it is strongly expressed in all cell types at all times in development. Other attractive features of this gene are that it is expressed in the cytosol, and that regulation is at the transcription level (Punt et al, 1988). This promoter has previously been used to drive the expression of *lacZ* (Punt et al, 1990), and other heterologous genes (e.g. phleomycin resistance; [Punt, 1992]).

1.5 AIMS AND OBJECTIVES OF THIS STUDY.

The aim of this project was to develop a new fungal GUS expression vector with a multi-cloning site and to assess its properties relevant to uses in reporter gene studies.

The steps to be taken in the project were:

- Construction of the GUS expression vector containing a multi-cloning site,
- Testing of the vector by insertion of the constitutive promoter, *gpdA*,
- Evaluation of its performance in cotransformation using the dominant selectable marker, phleomycin resistance,
- Testing the physiological impact of the presence of the vector DNA in transformation,
- Assessing the relationship between transformed gene copy number and GUS expression levels in the transformants.